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(54) **Hyper-sensitivity related gene.**

(57) The hsr203J gene of SEQ ID No. 1 and individual components thereof including its promoter and regulatory regions thereof, its coding region, its gene product ; modifications thereto ; applications of said gene, promoter region, regulatory region and coding region and modifications thereto ; DNA constructs, vectors and transformed plants each comprising the gene or part thereof.

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This invention relates to an hsr (hypersensitivity-related) gene family and individual components thereof including its promoter and regulatory regions thereof, its coding region, its gene product; modifications thereto; applications of said gene, promoter region, regulatory region and coding region and modifications thereto; DNA constructs, vectors and transformed plants each comprising the gene or part thereof.

The hypersensitive reaction (HR) of higher plants is a local inducible response associated with disease resistance to a pathogen. This response is characterized by a rapid and localized necrosis of tissues invaded by an incompatible (avirulent or non-host) pathogen, which prevents further spread of the invading microorganism. Several defense genes whose products may intervene in this plant response have been extensively studied: they include enzymes of the phenylpropanoid pathway involved in the synthesis of antimicrobial phytoalexins, enzymes with hydrolytic activities, toxic compounds and cell wall proteins. In infected plants, these genes are induced around the necrosis, once it has developed, i.e. late during the HR. Moreover, most of them are also strongly expressed during compatible interactions leading to the disease of the plant, and for some of them, during the normal development of the plant. The lack of specificity of these defense genes as well as their activation in the late steps of the HR suggest that they may not account by itself for establishment of the complex inducible response that is the HR, but rather may accompany this reaction. To date, the molecular mechanisms leading from plant-pathogen recognition to development of the HR are not known. In the "gene for gene" hypothesis, the initial step of plant-pathogen recognition leading to resistance involves the putative interaction between the products of a plant resistance gene and of the corresponding pathogen avirulent gene. Genetic studies indeed revealed that the outcome of many plant-pathogen interactions are determined through single dominant genes in both partners. Several rapid physiological changes have been also associated with the HR, such as electrolyte leakage, changes in respiration rates and more recently oxidative cross-linking of cell-wall proteins. However, in no case has a plant gene been described whose activation is specific or at least preferential during the resistance reaction, and precedes the development of the HR.

It is known that *Pseudomonas solanacearum*, a vascular bacterium, causes a lethal wilting of different plant species including *Solanaceae*. In this bacterium, a hypersensitive response (hrp) and pathogenicity gene cluster has been shown to control both the ability to elicit the HR on non-host plants and to cause the disease on host plants. In particular, hrp gene mutants of *P. solanacearum* have lost the ability to elicit an HR on tobacco plants. Recently, it was established that the hrpN gene of the hrp gene cluster of another bacterial pathogen, *Erwinia amylovora*, encodes a proteinaceous HR elicitor called harpin. This result confirms the important role of hrp genes in eliciting the HR. Upon infiltration of tobacco leaves by an HR-inducing incompatible isolate, six different gene families were characterized which are activated early during the interaction, before any necrosis of the leaf was detected. These genes which were not induced upon infiltration by an hrp-isolate differed by the accumulation levels of their transcripts during the incompatible versus the compatible interactions: the str (sensitivity-related) genes are expressed to a similar extent in both types of interactions, whereas the hsr genes are activated preferentially during the HR.

The present invention relates to an hsr gene family represented by a gene, hereinafter designated hsr203J, the sequence of which is depicted in SEQ ID No. 1. The putative protein product (SEQ ID No. 2) of the gene exhibits little, if any substantial homology with known proteins. Tests employing i.a. the promoter region of the hsr203J structural gene operably linked to a reporter gene in transient gene expression assays and in transgenic plants indicate that the expression of the hsr203J gene is closely related to the development of hypersensitivity: the promoter is specifically activated during the HR several hours before the appearance of the necrosis, and the localization of its activation is restricted to cells inoculated with an incompatible bacterial isolate.

According to the present invention there is provided a recombinant DNA sequence including a region comprising the nucleotide sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

Hereinafter where the term "functional equivalent" is used in respect of the protein encoding region of the DNA sequence the term means the said region wherein one or more codons have been replaced by their synonyms, ie codons which specify a corresponding amino acid or a corresponding transcription termination signal.

Where the term "functional equivalent" is used in respect of transcriptional regulatory regions of the sequence the term means the said region wherein one or more nucleotides have been replaced by different nucleotides and/or the region wherein one or more nucleotides have been added or removed with the proviso that the thus produced equivalents retain transcriptional regulatory activity and exhibit substantial homology with the region, or part thereof, which is 5' to the above mentioned protein encoding region.

As used herein, the term "substantial homology" refers to a DNA sequence which hybridizes under conventional hybridization conditions with a reference sequence. Preferably the hybridization conditions refer to hybridization in which the TM value is between 35 and 45°C. Most preferably the term substantial homology

rafars to a DNA saquanca which hybridizas with the refarance sequanca undar stringent conditions (as dafinad below).

Tha term "regulatory region" as used harain refers to the nucleotida region in tha sequence depicted in SEQ ID No. 1 which is 5' to tha protain ancoding region in the sequance. Tha regulatory region thus includes the promoter of the hsr203J gene and the functional components of the promoter which affect transcription. Such functional componants include a "dalation promoter" and transcriptional "silencers" and "anhancars".

A "deletion promoter" within the context of the present invention is any hsr203J derived promoter which has a deletion relative to the natural promoter and which still retains promoter activity. Such promoter activity may ba anhanced or substantially tha sama when comparad to tha nativa promotar. Tha skillad man is awara of the manner in which deletion promoters can be assayed for retention of their promoter activity. Deletion promoters according to tha present invantion are inducibla, *intar alia*, by plant pathogans, and find utility in constructs comprising structural ganes providing for improved diseasa resistance.

Whera tha tarm "functional aquivalent" is used in connection with a protain, tha sequanca of which is dictatad by at laast a part of tha DNA sequance depicted in SEQ ID No. 1, tha tarm means a protein having a lika function and lika or improved specific activity, and a similar amino acid sequence. Tha present invantion includas pura protains which hava an amino acid saquance which is at laast 60% similar to tha saquanca or part (sea below) thereof of tha protain depicted in SEQ ID No. 2. It is preferrad that tha degree of similarity is at least 60%, more preferred that the degree of similarity is at least 70% and still more preferred that the degree of similarity is at least 80%.

In tha contaxt of the present invantion, two amino acid sequences with at least 60% similarity to each other are defined by having at least 70% identical or similar amino acids residuas in the same position when aligned optimally allowing for up to 4 deletions or up to 10 additions. For the purpose of the present invantion:

Alanine, Sarina and Thraonina are similar;

Glutamic acid and Aspartic acid ara similar;

Asparagine and Glutamina are similar;

Arginina and Lysina are similar;

Isoleucina, Leucina, Methionine and Valina are similar;

Phenylalanina, Tyrosine and Tryptophan ara similar.

Whara tha tarm "part" is usad in connaction with a protain saquance, tha term maans a paptida comprisad by tha saquence depicted in SEQ ID No. 2 and having at least 5 amino acids. More preferably the peptide has at least 20 amino acids, and still more preferably the peptide has at least 40 amino acids.

Whera tha tarm "part" is used in connection with a nucleotida saquanca, tha tarm means a nucleotida sequence comprisad by the sequance depicted in SEQ ID No. 1 and having at least 15 nuclaotidas. Mora prefarably tha part has at least 25 nuclaotidas, and still more preferably the part has at least 40 nucleotidas.

The invention also includes a recombinant DNA sequenca including a region comprising nuclaotides 1413 to 2417 of tha sequence depicted in SEQ ID No. 1 or a functional aquivalent thereof, or a recombinant sequence comprising a part of said region or said aquivalant. Nucleotidas 1413 to 2417 correspond to tha protein-encoding region of the hsr203J gana which is useful in that tha gena product has a functional role in regulating or providing for disaase rasistance in plants. Thus, tha protein coding saquanca, or a part thereof, of tha hsr203J gena may ba fused to an inducibla promoter such as that regulating axpression of WIN, WUN or PR-proteins so that upon infection by a compatibla pathogan, expression of tha hsr203J structural gena is induced. Tha ansuing activation of tha hyparsansitiva rasponsa by tha hsr203J protain in infactad plant cells halts furthar spread of tha pathogan.

The invention also includes a recombinant DNA sequence including a region comprising nucleotides 1 to 1341 of tha saquance dapictad in SEQ ID No. 1 or a functional aquivalant thereof, or a racombinant sequance comprising a part of said region or said aquivalant. Nucleotides 1 to 1341 correspond to tha non-protain ancoding ragion of tha saquance which is 5' to tha said protain ancoding ragion. The region of tha said DNA sequence comprising nucleotides 1 to 1341 includes tha transcriptional regulatory region of tha hsr203J gana, including tha promotar (binding site for RNA polymarasa) and transcriptional silancers and anhancers.

Silancer and enhancer alamants are usaful in that thay anabla modulation of tha leval of axpression of the structural genes under thair control.

Tha invantion still furthar includes a racombinant DNA saquance including a region comprising nuclaotides 1 to 651 of the sequence dapicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent. The region comprising nucleotides 1 to 651 includes a transcriptional silancer.

Tha invention still further includes a recombinant DNA sequence including a region comprising nucleotides 652 to 1341 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent. Tha region comprising nucleotidas 652 to 1341

includes a transcriptional enhancer and the promoter (ie RNA polymerase binding site) of the hsr203J gene.

The invention further provides the use of hsr203J promoter sequences as affinity substrates for the identification and subsequent purification of hsr203j promoter binding proteins (hsr-PBP's) and proteins associated with these hsr-PBP's. Such hsr-PBP's have been partially characterized, are probably present constitutively and may bind to hsr203J promoter sequences upon incompatible reaction of the host plant such as occurs when *Nicotiana tabacum L.* is inoculated with specific strains of *Pseudomonas solanacearum*.

The invention still further includes a recombinant DNA sequence including a region comprising nucleotides 1195 to 1341 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or recombinant sequence comprising a part of said region or said equivalent. The region comprising nucleotides 1195 to 1341 includes a bacterial response element which is capable of binding to specific proteins which are produced by pathogens during their infection of tissue, and which are implicated in the development of the hypersensitive response (see above).

The invention still further includes a recombinant DNA sequence including a region comprising nucleotides 1195 to 1268 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent. This region more precisely defines the bacterial response element.

The invention still further includes a recombinant DNA sequence as disclosed above wherein the said region, part or equivalent thereof is located on the 5' side of, and is operably linked to, a protein-encoding sequence of a heterologous gene or to a sequence comprising nucleotides 1413 to 2417 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof. It is particularly preferred that a translation enhancing sequence is present between the region or part or equivalent thereof, and the protein-encoding region of the DNA sequence 3' thereto.

The heterologous gene may be any suitable structural gene, including a selectable or screenable marker gene or a gene, the product of which is capable of conferring resistance or tolerance to at least one of the following: insects, herbicides, fungi, bacteria and viruses, a marker gene for use in disease pressure forecasting and anti-feedant genes.

The promoter, and/or regulatory regions of the hsr203J gene may be fused to a structural gene encoding a non-diffusible cytotoxic gene product such as an ribonuclease, protease, lipase or glucanase. Induction of expression of such structural genes provides a rapid and localized response to infection by pathogens, and may be useful in providing resistance or improving tolerance of the plant to the pathogen.

Moreover, the regulatory regions of hsr203J gene may be used in the creation of "detector" plants enabling the early detection of disease pressure. The hsr203J promoter and/or regulatory regions thereof, may be fused to a nucleotide sequence providing for a visual alteration to the host plant phenotype upon activation of the promoter by infection. Such sequences include the anti-sense orientation of the gene encoding the Small Sub-unit of Ribulose B-phospho Carboxylase (SS-RUBISCO) which causes localized bleaching of green tissues. Such sequences could also encode a gene encoding a key enzyme in pigment biosynthesis such as chalcon synthase.

The invention also includes recombinant DNA according to the invention, which is modified in that codons which are preferred by the organism into which the recombinant DNA is to be inserted are used so that expression of the thus modified DNA in the said organism yields substantially similar protein to that obtained by expression of the unmodified recombinant DNA in the organism in which the protein-encoding components of the recombinant DNA are endogenous.

The invention still further includes a DNA sequence which is complementary to one which, under stringent conditions, hybridizes to any one of the above disclosed recombinant DNA sequences.

"Stringent hybridization conditions" are those in which hybridization is effected at between 50 and 60°C in 2X saline citrate buffer containing 0.1%SDS followed by merely rinsing at the same temperature but in a buffer having a reduced SCC concentration which will not affect the hybridizations that have taken place. Such reduced concentration buffers are respectively (a) 1xSCC, 0.1%SDS; or (b) 0.5xSCC, 0.1%SDS; or (c) 0.1xSCC, 0.1%SDS.

The invention still further includes a DNA vector comprising a recombinant DNA sequence according to the invention or a DNA sequence which is complementary to one which, under stringent conditions, hybridizes thereto.

It is preferred that the vector according to the invention be used to transform a eukaryotic host, preferably of plant origin. It will be appreciated that suitable micro-organisms may be transformed with such a vector, and such micro-organisms represent yet a further embodiment of the invention.

The term "plant" is used herein in a wide sense and refers to differentiated plants as well as undifferentiated plant material such as protoplasts, plants cells, seeds, plantlets etc. that under appropriate conditions can develop into mature plants, the progeny thereof and parts thereof such as cuttings and fruits of such

plents.

Preferred vectors will of course vary depending on the chosen host. For dicotyledons, the vector may be introduced into e protoplest by contacting the vector with the protoplast in a suitable medium and under appropriate conditions which render the protoplast competent for the uptake of DNA; the vector mey elso be employed in the form of an *Agrobacterium tumefaciens* Ti-plasmid derivative which infects plant cells or protoplasts. Monocotyledons ere preferably transformed by micro-injection, electroporation or by use of the microprojectile gun, using the so-called ballistic technique. In eny case, appropriate transformation vectors and protocols are well known in the art. The transformed cells or protoplests are cultured in en appropriate culture medium, end a transformed plant is regenerated in a manner known *per* se. The introduced nuclear material is stably incorporated into the genome of the regenerated transformed plents which accordingly express the desired genes.

Exemples of genetically modified plants according to the present invention include: fruits, including tomatoes, peppers, mangoes, peaches, apples, pears, strawberries, banenes, and melons; field crops such as canole, sunflower, tobacco, suger beet, small grain cereels such es wheet, berley end rice, corn end cotton, and vegetables such as potato, carrot, lettuce, Brassica oleracea such as cabbege and onion. The particulerly preferred plants are suger beet end corn.

The invention still further includes the progeny or seeds of such plants, and the seeds and progeny of said progeny.

The invention still further includes protein obtained by expression of the recombinant DNA according to the invention, end in particular, expressed protein having the emino acid sequence depicted in SEQ ID No. 2, or e pert thereof or e functionel equivelent of seid sequence or part.

The Invention will be further epperent from the following description, and the associated Figures end Sequence Listings.

Of the Figures:

FIG. 1 shows e chimeric construct used for transient gene expression assays in tobecco protoplasts end for transformation of tobecco plents via *Agrobacterium tumefaciens*. Fig (A) shows the restriction map of the chimeric β-glucuronidase gene on pHG21 (or pHG21A). This gene consists of e translationel fusion between 1.4 kb of the 5' flenking sequence from the hsr203J gene and the coding region of uidA gene linked to the nopaline synthese gene polyadenylation signel (nos T). Fig (B) shows the sequence (SEQ ID No. 2) of the pHG21 trensletionel fusion joint. The hsr203J gene sequence is in bold type end the *uidA* sequence is in standerd type. The orientation is 5' to 3', and the arrow in the Figure indicates the position of the fusion between the sequences.

FIG. 2 shows the effect of infection with different isolates (hrp, K 60 and GMI 1000) of *Pseudomonas solanacearum* on hsr203J promoter activity in transformed tobacco protoplests. As a control, water was added to the protoplests. Plasmids pBI201 end pBI221 are respectively negetive end positive control plesmids; pHG21 is the hsr203J-uidA gene fusion. GUS activity assays were performed 24 h after incubation. The deta shown represent the meen of three separete experiments.

FIG. 3 shows the time course of hsr203J promoter activation of the GUS gene in transgenic tobecco leaves infiltrated with different isoletes (hrp, K 60 end GMI 1000) of *P. solanacearum*. GUS activity was measured in extracts of four leeves from two pHG21-14A transforments.

FIG. 4 shows a quantitative analysis of GUS activity in locally bacterial-infected transgenic tobacco plants. Figure (A) shows the induction of β-glucuronidese activity in the inoculeted third leaf, end in the upper end lower un-inoculated leeves. Figure (B) shows the induction of β-glucuronidase ectivity in end around the lesion of the inoculeted third leaf. The following tissue samples were assayed: lesion meening necrotic tissue resulting from the wounding end/or bacteriel infection; 0-3mm meening apparently healthy tissue up to 3mm from the lesion; 3-6mm meaning apperently healthy tissue 3 to 6mm surrounding the lesion. Inoculation wes performed on pHG21-14A trensforments. Smell leaf perforations were covered by e droplet of the becteriel suspension (3 μL containing 10^8 c.f.u./mL) or water, as indicated on the Figure. Tissue samples were collected 18 h after inoculetion.

FIG. 5 shows the effect of hrp mutants on the activetion of hsr203J promoter in transgenic pHG21(14A) tobacco plants. Figure (A) shows localization of hrp mutations in the different transcription units of the hrp gene cluster. Figure (B) shows measurements of GUS activity in leaves at 18 h after inoculation by the hrp K60 or GMI 1000 isoletes or by water, or by the hrp mutants indicated in Figure (A). Inoculetion was performed es described for Figure 4.

FIG. 6 shows schematically the construction of plasmids pHGD heving several deletions of pHG21.

FIG. 7 shows in transgenic tobacco plents the expression of the GUS gene by constructs obtained by

5' promoter deletions of pHG21 (according to the scheme of Figure 5). The plants were transformed with 5 µg DNA, and the value 100 was given to the GUS activity obtained by transformation with the pHG21 construct. The Figure shows the increase in activity (after 18 hours) of the GUS gene as a consequence of infiltration of the transformed plants with the bacterial strains Delta 3, K60 and GMI 1000. As controls plants were infiltrated with water.

Of the Sequences:

SEQ ID No. I shows the nucleotide sequence of the hsr203J gene, including the protein encoding region and promoter and transcriptional regulatory elements therefor, isolated from tobacco. The protein coding region of the gene is comprised by nucleotides 1413 to 2417 in the sequence. Putative polyadenylation signals are present 3' to the protein coding region of the gene and the sequence responsible for the HR is within about 1.4 kb of the 5' non-coding region of the gene. In essence the sequence comprises:

a) a 72 bp mRNA leader sequence, located at nucleotides 1341 to 1412 inclusive;

b) CAAT and TATA consensus sequences located at nucleotide positions 1282-1286 and 1313-1316 respectively;

c) the translation start site codon at nucleotide positions 1413-1415;

d) the "deletion promoter" sequence located at nucleotides 1-1341 inclusive which is substantially responsible for the promoter activity;

e) the sequence located at nucleotide positions 1195-1268 having an enhancing effect on the promoter activity;

f) the sequence located at nucleotide positions 1-651 having a silencing effect on the promoter activity.

SEQ ID No. 2 shows the translation product of the hsr203J structural gene, encoded by nucleotides 1413-2417 in SEQ ID No. 1;

SEQ ID No. 3 shows a linker region for a chimeric gene comprising the 5' flanking region of the hsr203J structural gene and the coding region of the uidA reporter gene. The start codon for the hsr203J structural gene is at nucleotides 10-12 in the sequence and nucleotides 13-64 encode the N-terminal sequence of the hsr203J gene product.

Bacterial Strains and Plant Material

The source of the *Pseudomonas solanacearum* strains used herein is depicted in Table 1.

TABLE 1

***Pseudomonas solanacearum* wild type and mutant strains used in this study, and their ability to induce symptoms on tobacco**

Strains	Source or reference	Isolated from	Tobacco response
Wild type			
GMI 1000	Boucher *et al.* (1)	Tomato	HR
K60	Lozano *et al.* (17)	Tomato	Disease
Mutants derived from GMI 1000 (deletion of hrp gene cluster)			
Δhrp	Boucher *et al.*, unpublished		No symptoms
Mutants in hrp gene cluster derived from GMI 1000 (Tn5-B20 mutagenesis)			
GMI 1462, 1475, 1494, 1492, 1487	Arlat *et al.* (18)		No symptoms
GMI 1423, 1425	Arlat *et al.* (18)		Partial and/or delayed HR
Mutant derived from GMI 1000 (Tn5-B20 mutagenesis outside the hrp gene cluster)			
GMI 1485	Arlat *et al.* (18)		HR

The GMI1000 and K60 isolates are wild-type *P. solanacearum* strains, the former induces the development of an HR on tobacco leaves within 24 h after infiltration, and the latter causes the typical lethal wilting disease. A derivative of the GMI1000 isolate, called *Δhrp*, deleted for the *hrp* gene cluster, causes no apparent symptoms in inoculated leaves. Eight mutant strains derived from GMI1000 by transposon Tn5-B20 mutagenesis were used as described below. The GMI1462, 1475, 1494, 1485, 1423 and 1425 strains are each mutated in one of the six putative transcription units of the *hrp* gene cluster. All these strains have lost the ability to cause an HR on tobacco, except strains GMI1423 and 1425 which are mutated in the right-hand end of the *hrp* gene cluster, and induce only a partial and/or a delayed HR on tobacco; and the strain GMI1485 which is mutated outside of the *hrp* gene cluster and elicits a normal HR on tobacco and constitutively expresses the structural gene of β-galactosidase. All these are grown at 28°C in B or BGT media (1). The cultivars of *Nicotiana tabacum L.* used herein: Bottom Special and Samsun, exhibit similar responses after bacterial inoculation. The seedlings are grown *in vitro* on Murashige and Skoog (MS) medium (2) during 4 to 5 weeks (25° C, 16 h photoperiod, 15 Watt/m²), and then transferred to soil in a growth chamber (25°C, 16 h photoperiod, 30 Watt/m²).

Isolation of hsr203J gene, and nucleotide sequence analysis

A tobacco *(Nicotiana tabacum L.* cultivar NK326) genomic library constructed in the bacteriophage λ-EmbI3 (Clontech) is screened with the pNt203 cDNA clone (3). The PstI insert of pNt203 is labeled by the random primer technique (4). Replicate nitro-cellulose filters of the genomic library are treated and hybridized as suggested by the manufacturers (Amersham). Four different genomic clones including hsr203J are isolated. Exonuclease III deletions are performed at both ends of DNA inserts subcloned in the phagemid pKS (Stratagene) according to Henikoff (5), and both strands are sequenced by the dideoxy chain termination method (6) using Sequenase (US Biochemical, Corp.). Sequence compilation and analysis are performed by using the Genetics Computer Group software of the University of Wisconsin (7). Homology searches with the Genebank (release 71.0) and Swissprot (release 21.0) data bases are performed using the FASTA algorithm (8). The protein sequences are analysed for potential N-terminal signal sequences and membrane-spanning domains using release 5.0 of the PC/Gene Programme (Department of Medical Biochemistry, University of Geneva, Swit-

zerland). The transcription start site is determined by the primer extension technique using polyA+ RNA extracted from tobacco leaves 9 hours after inoculation with the incompatible isolate and an oligonucleotide located at the ATG codon (nucleotides 1413 to 1415 in SEQ ID No. 1).

Reporter gene constructs

A 2.2 kilobase (kb) BglII fragment containing 1.3 kb of the 5' non-coding region of the tobacco hsr203J gene and 890 base pairs (bp) of the nucleotide sequence downstream of the transcription start site is cloned into the BamHI site of phagemid pKS, to produce pKJ2.2. This plasmid is digested with BstBI, which cuts once 55 bp 3' of the hsr203J translation initiation codon, and the BstBI generated ends were blunt end ligated by the Klenow fragment of DNA polymerase before digestion with SalI. This 1.5 kb SalI - BstBI fragment is cloned into the SalI - SmaI site of the β-glucuronidase (GUS) expression binary vector pBI101.2 (9) to produce the hsr203J- uidA gene fusion pHG21A. A 3.5 kb HindIII - EcoRI DNA fragment of pHG21A, including the hsr203J promoter and the uidA coding sequence, is ligated into the HindIII - EcoRI digested pUC19 vector to produce pHG21, for transient expression gene assays (Fig. 1). The pHG21 and pHG21A constructs therefore contain 1341 bp 5' non coding sequence, the 72 bp leader sequence, the first 55 bp of the coding sequence of hsr203J fused in frame with the GUS coding sequence, and the nopaline synthase (nos) gene polyadenylation signal. The translational fusion is confirmed by direct double-stranded sequencing with a GUS specific primer (10). Two additional plasmids, pBI201 and pBI221, contain respectively a promoterless uidA gene, and a cauliflower mosaic virus (CaMV) 35S promoter - uidA gene, upstream of the nos terminator, in the pUC19 vector (Clontech).

Protoplast isolation and transient expression assays

Leaves of 4 to 5-week-old in vitro grown tobacco plants, cultivar Samsun NN, are used for isolation of protoplasts by incubating leaf sections in TO medium (11) containing 1 g/L cellulase R10 Onozuka, 200 mg/L macerozyme Onozuka (Yakult Honsha, Nishinomiya, Japan) and 500 mg/L pectolyase Y23 (Seishin Pharmaceutical Ind.), for 15 h at 22° C in darkness. Protoplasts are separated from the cellular debris by sieving through an 85 µm nylon mesh followed by centrifugation at 50 g for 5 min onto a 1 mL cushion of 19 % (w/v) sucrose. Floated protoplasts are washed once with TO medium, counted, and adjusted to the density of 1.5×10^6 protoplasts/mL. Transformation is performed by incubating the protoplasts (320 µL samples) at 45° C for 5 min, after a brief cooling at room temperature, by adding plasmid DNA (50 µg per assay in 10 mM Tris-HCl, pH 8) and 160 µL of a PEG solution (40 % PEG, 0.4 M mannitol, 30 mM $MgCl_2$, 0.1 % Mes pH 5.8). Protoplasts are gently mixed for 10 min at room temperature. They are then collected by centrifugation and resuspended in 500 µL TO medium. The bacterial suspension (10 bacteria/protoplast) prepared as previously described (12) is then added. After incubation at 28° C for 24 h, the protoplasts are lysed by the addition of 50 µL of 10X GUS buffer, centrifuged and the supernatant is assayed for GUS activity (10).

Transgenic tobacco plants

pHG2A, pBI121, and pBI101 are mobilised from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* strain LBA 4404 (13) and transgenic tobacco plants (N. tabacum, Bottom Special) are generated by the leaf disk method (14). Transformed plants are selected on MS medium containing 0.8 % Difco agar, kanamycin at 100 µg/mL and carbenicillin at 500 µg/mL. Transgenic plants are self-fertilized and seeds are collected. Their genotypes are determined by progeny (T2) analysis, by germination on MS medium containing kanamycin (500 µg/mL).

Inoculation of transgenic plants with bacterial isolates

All the inoculation experiments are performed on kanmycin-resistant T2 plants, with at least 2 plants of the same genotype per experimental condition. For the screening of transformants and kinetic experiments, tobacco leaves are detached from 8 week-old plants and infiltrated in vacuo with the bacterial suspension (10^7 c.f.u./mL) or water as described in ref (12). Syringe infiltration experiments are performed on 8 week-old plants by infiltrating the bacterial suspension (10^8 c.f.u./mL) into a small region of undetached leaves with a syringe without a needle. For some experiments, inoculations were performed on 5 week-old plants grown in Magenta cubes (Sigma) on MS medium. Each half of a leaf is perforated 6 times with an 10 µL-Hamilton needle and a 3 µL droplet of bacterial suspension (10^8 c.f.u./mL in 0.4 % Difco agar) is immediately deposited at the wounded sites.

For localized root inoculation, 4 week-old plants are grown on a raft (Sigma) in contact with MS medium containing 0.2 % Difco agar, and inoculated with a 3 µL droplet of bacterial suspension through a wound made with a needle at one centimeter from the root apex or at a secondary root emergence. For generalized root inoculation, the whole plant is detached carefully from the raft, avoiding wounding, and the root system is immersed in 7 mL of the bacterial suspension (10^8 c.f.u./mL).

Inoculated plants are maintained at 28° C, and analysed either directly or stored at -80° C after incubation time.

GUS assays

Plant tissue is ground in liquid nitrogen, homogenized in 1X GUS buffer, centrifuged for 5 min at 10,000g and the supernatant assayed for GUS activity, as previously described (15). Protein concentration is determined using the Bradford dye reagent. GUS activity is expressed as picomoles of 4-methylumbelliferone per min per mg of protein. Alternatively, histochemical assays are performed on fresh tissue using X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide, Clontech) or Magenta-gluc (Biosynth AG) as the substrate (10). For some experiments, samples are fixed in 0.3 % formaldehyde/ 50 mM $NaPO_4$ buffer pH7, then cleared by boiling in ethanol and stored in ethanol 70 %.

β-galactosidase assays

Following the GUS histochemical assay, some samples are equilibrated in Z' buffer (18) (100 mM $NaPO_4$ buffer pH 7.4, 10 mM KCl, 1mM $MgSO_4$), fixed in 1.25 % glutaraldehyde for 1 h in order to inactivate endogenous plant β-galactosidases, rinsed and stained at 28° C with 0.8 mg/mL Magenta-Gal (Biosynth Ag) or X-gal in Z' buffer containing 5 mM $K_3FeCN)_6$ and 5 mM $K_4Fe(CN)_6$, then cleared by boiling in ethanol and observed by dark- or bright-field microscopy.

Characterization of hsr203J gene

The hsr203J gene is isolated by screening a genomic tobacco library with pNt203 cDNA clone. It belongs to a small multi-gene family consisting of a minimum of 4 genes (see ref. 3) and at least 2 genes of this family corresponding to 2 different cDNA clones (pNt203 and pNt239) are expressed during the HR.

Sequence analysis of the 2.7 kb DNA region of hsr203J (SEQ ID No. 1) reveals a single open reading frame (ORF) with no intron and a potential coding capacity of 355 amino acids. The nucleotide sequence of the said 2.7 kb region is identical to the pNt239 cDNA clone except for 2 substituted bp (not shown). These mismatches are probably due to the isolation of the genomic and cDNA clones from different tobacco cultivars: the genomic clone is isolated from cultivar NK326 whereas the pNt239 cDNA clone is obtained from the cultivar Bottom Special. The predicted hsr203J structural protein (SEQ ID No 2) has a Mr. of 37.5 kDa and a theoretical isoelectric point of 5.17.

The transcription start site is mapped by primer extension to a position 72 bp upstream of the putative translation initiation codon. The promoter and 5'-untranslated region exhibited no obvious sequence homology to cis-elements already described in defense genes.

Transient expression of the hsr203J-uid A gene fusion in tobacco protoplasts

pHG21 plasmid is composed of a translation fusion between 1.4 kb of the 5' flanking sequence from the hsr203J gene and the coding region of the uidA reporter gene, linked to the 3' untranslated region of the nopaline synthase gene (Fig. 2). The plasmids pBI201 and pBI221 are used respectively as negative and positive controls in transient assays.

Initial experiments show that protoplast viability as quantified by Evans blue exclusion is not significantly altered in the presence of bacteria at 10 to 100 bacteria per protoplast (data not shown). Subsequently experiments are performed with 10 bacteria per protoplast. At this bacterial density, the expression of GUS fused to the hsr203J promoter in response to GMI1000 isolate is 6-fold higher than in response to the controls (water or *Δhrp* inoculation) (Fig. 3). In comparison, inoculation with the compatible isolate, K60, led to a 2-fold increase in enzyme activity. These levels of GUS activity have to be compared with those measured in protoplasts transformed with the CaMV 35S-uidA gene fusion (pBI221) which exhibit a high and almost constitutive level after the various inoculation treatments (Fig. 3).

The results of transient assays therefore indicate clearly that the hsr203J promoter contains all the necessary elements for its preferential activation by the HR-inducing bacterial isolate, and that this expression

system perfectly mimics the plant/pathogen interaction.

Expression of hsr203J-uid A gene fusion in transgenic tobacco

In order to determine the spatial and temporal patterns of expression of the hsr203J promoter in planta, the hsr203J-uidA gene fusion is transferred to tobacco by leaf disk transformation. T2 plants resistant to kanamycin are used in all the experiments. Of 23 kanamycin resistant transformants, 20 expressed the gene fusion and these all exhibit the same overall pattern of expression: GUS activity is found maximal after infiltration with GMI1000, with a 2- to 90-fold stimulation over control infiltrations (water or *Δhrp)*, and a 2- to 25-fold induction over K60 infiltration, 18 hours after inoculation (not shown). These levels are comparable to those obtained in transient experiments after inoculation by GMI 1000 or K60.

Based on this analysis, a transformant (pHG21-14A) which displays a 90-fold stimulation of GUS activity after incompatible inoculation compared to control infiltrations, and contains one insertion of the gene fusion per haploid genome, is selected. The presence of a native gene fusion is checked by Southern analysis of genomic DNA (not shown).

Assay of extractable GUS activity and GUS histochemical localization are both used to monitor the activity of the hsr203J promoter in different organs during plant development and in response to bacterial inoculation. No GUS activity was detected in 4, 7 or 15 day-old pHG21-14A tobacco seedlings, either in healthy leaves, or in flowers of fully grown plants (data not shown). These data indicate that the hsr203J promoter is strongly activated in leaves inoculated with the HR-inducing isolate, GMI1000, 18 h after infiltration, as indicated by the screening of all the transformants obtained. A kinetic study is performed on transformant pHG21-14A (Fig. 3), which shows that in leaves infiltrated with GMI1000, GUS activity increases to a level 12-fold over control values 6 h after inoculation, reaches a maximum of 200-fold stimulation at 9 h, and decreases to an intermediate level (80-fold induction) upon longer incubations. Much lower levels are measured after K60 infiltration, and undetectable levels of GUS activity were found in leaves infiltrated with water or the *Δhrp* isolate at any incubation time.

Plants transformed with the promoterless construct pHI101 show negligible levels of GUS activity. Moreover, plants transformed with pBI121, which contain a CaMV 35S-uid A gene fusion, show similar levels of enzyme activity, whatever the nature of the inoculum (not shown). Thus the hsr203J-uid A gene fusion exhibits a distinct and specific pattern of activation upon bacterial inoculation of transgenic tobacco plants that closely matches the *in vivo* pattern of accumulation of hsr203J transcripts in infiltrated tobacco leaves (3). These results also indicate that hsr203J promoter is early and specifically activated during an incompatible plant/pathogen interaction, and that its induction is *hrp* gene-dependent since the bacterial isolate which is deleted of hrp genes is unable to activate the hsr203J promoter.

Localization of hsr203J-uidA activation in response to bacterial inoculation

Different inoculation tests are performed on transformants pHG21-14A in order to localize precisely hsr203J promoter activation in response to bacterial inoculation; first, in tobacco leaves in order to investigate promoter induction during a typical HR, and secondly, in roots, which are the organs naturally infected by the bacteria.

Leaf inoculations:

In order to test whether the hsr203J-GUS gene expression is local or systemic, leaves of 5 week-old transgenic plants are inoculated with bacterial suspension droplets. After incubation for 18 and 70 hours, GUS activity is determined in half of the inoculated leaf as well as in upper and lower leaves. The results show a 15-fold induction of this activity in the inoculated leaf, whereas very low levels are detected in the lower and upper leaves (Figure 4A). The other half of the inoculated leaf is used for histochemical GUS assay. A narrow blue-stained region is visualized 18 h and 70 h after inoculation with the HR-inducing bacterial isolate, surrounding the wounded area, which is restricted to a few cell layers and is localized very close to yellowing, probably dead, cells. The intensity of the staining increases 70 h after inoculation. Only a few dispersed cells exhibit a faint blue staining after K60 inoculation; water or Δhrp isolate inoculations induce no detectable GUS expression. Staining of transgenic plants harboring a chimeric uidA gene under the control of the CaMV 35S promoter results in the staining of the entire leaf, with no preferential staining around the lesions, thus demonstrating the specific nature of the induction of the hsr203J promoter in this area. A more detailed localization of this activation during infection is provided by GUS activity measurements in small squares surrounding the lesion, 18 h after inoculation (Fig. 4B). High levels of enzyme activity (48-fold stimulation over control values) are found

only within the necrotic lesion itself after inoculation by GMI1000. No detectable enzyme activity is found in tissue up to 3mm away from the lesion.

In order to determine how early the hsr203J promoter is activated in the inoculated area, histochemical GUS localizations are performed on leaves of 8 week-old transgenic plants locally infiltrated by a syringe with the bacterial suspensions or K60. As early as 6 h after inoculation by the GMI1000 isolate at which time there is no visible tissue necrosis, the leaf infiltrated area shows a blue staining whose intensity increases 9 h after inoculation. At later incubation time points, a yellow necrosis progressively appears, limited on its border by a thin blue area still located within the infiltrated part of the leaf.

These different experiments show clearly that hsr203J-GUS expression is confined to a restricted area corresponding precisely to cell layers infected by the HR-inducing isolate, GMI1000.

Root inoculations:

Roots of transgenic plants grown on rafts are wounded and inoculated with a droplet of bacterial suspension. After 48 h incubation, histochemical localization of GUS activity is performed. Staining only observed in roots infected by GMI1000 extends from the initially inoculated site to a 2 mm distanca in the root. Cytological studies indicate that hsr203J promoter activation appears not to be cell-type dependent (not shown). A generalized root inoculation is also performed by simply immersing the whole root system in a bacterial suspension. In this case, GUS activity is found in restricted regions of the roots, i.e. at the point of origin of secondary roots. Expression of the gene fusion at this specific location has to be correlated with the existence of preferential sites of bacterial entry into the host which have been observed along the emergence sheath of secondary roots. At these specific sites, a double staining of GUS activity and bacteria by using a bacterial isolate containing a β-galactosidase fusion, shows a good correlation between the activation of the hsr203J promoter and the presenca of bacteria. A superficial and intercellular bacterial colonization of the root tips has also been observed and results in a strong activation of the hsr203 promoter in this part of the root.

Thus, the hsr203J-GUS gene fusion exhibits a distinct and specific pattern of activation in transgenic tobacco plants in response to bacterial infection and one which closely matches the pattern of bacterial ingress into the plant.

Dependence of hsr203J-uidA activation on *hrp* genes

Different *P. solanacearum* strains mutated in one of the six transcription units of the hrp gene cluster (Fig. 5A) are used to inoculate transgenic plants (pHG21-14A) by the droplet method. These mutant strains have lost the ability to induce an HR on tobacco, although two of them, GMI1425 and GMI1423, lead to a partial or delayed HR. 18 h after incubation, no effect on GUS activity can be detected with 6 out of 7 tested mutants; only GMI1423 leads to an increase in enzyme activity comparable to that of the wild type strain, GMI1000 (Fig. 5B). These data indicate that hsr203J activation requires almost a whole functional *hrp* gene cluster.

Until now, no plant gene has been identified which is specifically implicated in the perception of an incompatible pathogen, the transfer of that signal throughout the cell or finally the programmed cell death (HR) which provides an efficient mechanism for the limitation and eventual elimination of the pathogen.

The gene hsr203J (SEQ ID No. 1) is the first hypersensitivity-related gene to be isolated, whose promoter exhibits a rapid, high-level localized and specific activation in response to an HR-inducing bacterial isolate.

Construction of deletions of the 5' promoter region of pHG21

Unidirectional deletions of the promoter of the chimeric gene have been realized starting from the 5' end according to Henikoff (5). For that purpose, plasmid pHG21 (Fig. 1) is linearised employing the restriction enzymes ShpI and SalI, and then digested by exonuclease III. Constructions having successive deletions, each distant by ca. 200 pb, are selected. The localization of the 5' end of the deletion is determined by sequencing the region and comparison with the nucleotide sequence of the hsr203J gene (see Figure 6).

Effect of deletions on gene expression of the chimeric gene in transgenic tobacco plants

50 µg plasmid DNA corresponding to the different deletions (Fig. 6) are introduced by transformation into tobacco plants. The GUS activity is measured 18 hours after inoculation.

FIG. 7 shows the expression of the GUS gene by constructs obtained by 5' promoter deletions of pHG21 (according to the scheme of Figure 5). The plants were transformed with 5 µg DNA, and the value 100 was given to the GUS activity obtained by transformation with the pHG21 construct. The Figure shows the increase

in activity (after 18 hours) of the GUS gene as e consequence of infiltration of the transformed plants with the bacterial strains Delta 3, K60 and GMI 1000. As controls plants were infiltrated with water. These experiments indicate the presence of 2 main regions having e regulatory effect of the deletion promoter of the hsr203J gene.

One or more elements situated in the 1-651 nucleotide region of SEQ ID No. 1 are responsible for e diminution of the expression of the chimeric gene, and elements situated in the second region (nucleotides 652-1268) exhibit e positive effect on the activetion of the promoter of the hsr203J gene.

The study of the spatial and temporal patterns of promoter activation in roots and leaves of transgenic plants inoculated with *Pseudomonas solanacearum*, indicate thet

- the promoter is specifically activated during the HR several hours before the appearance of the necrotic lesion
- the localization of its activetion is restricted to the few cell leyers in contact with the becteria
- the promoter does not respond to verious stress conditions and is very weakly activated during compatible interactions
- the promoter ectivetion is strongly dependent on hrp (hypersensitive response end pethogenicity) genes of *Pseudomonas solanacearum*. These genes control the ability of the bacterium to elicit the HR in resistant or non-host plant and to cause the disease on the host plant.

In favour of a mejor role of the becterial hrp genes in the activation of hsr 203J gene promoter, is the fact that the hsr 203 promoter is expressed in response to an HR specific elicitor, herpin, product of one of the hrp genes of *Erwinia amylovora*. In response to this polypeptide, the promoter is activeted et similer levels to those observed with the corresponding evirulent strain, but more rapidly. Other potentiel inducers such es biotic and abiotic elicitors, resistance inducers, do not affect its expression. The generality of the specific expression of hsr 203J during incompatible interactions with becteriel pethogens hes been demonstrated by testing other pathogens such as *Pseudomonas syringae pv pisi / pseudomonas syringae pv tabaci*, and *Erwinia amylovora*.

In eddition the functionel enelysis of the cis elements responsible for the transcriptionel activetion of the hsr 203J gene in response to the incompatible bacterial strain, has been initiated by generating a series of 5' deletions and analysis of these constructs by transient assay and in transgenic plants. The results reveal the presence of a distal silencer element, and of two positive reguletory elements, one being quentitative (nucleotides 655-770 in SEQ ID No. 1), the other one being specific for the response to the bacterium, between nucleotides 1195 end 1268 of the SEQ depicted in SEQ ID No. 1.

These results indicate that the hsr 203J gene promoter exhibits new and original characteristics of activation with regerd to plant defense genes studied so far; its spetial end temporal program of ectivetion together with its specific induction during the HR underline the importance of this gene as e moleculer tool to study the establishment and regulation of the HR. In addition, a 74 bp sequence element has been defined as responsible for the inducibility of the promoter by the avirulent pathogen.

Although the invention has been specifically described with reference to activation of the hsr203J promoter in response to challenge of Tobacco plants with an incompatible pathogen, it will be appreciated that the promoter mey likewise be activeted by chellenge of other plents transgenic for the gene with other pethogens, including certain viruses and certain fungi, indicating thet specific expression of the hsr203J promoter is a general phenomenon of incompetible interactions between host end pethogen which lead to the hypersensitive response.

Moreover, the nucleotide sequence comprised by positions 1195 to 1268 of the sequence depicted in SEQ ID No. 1 containing the becteriel response element binds to nucleer protein extracts from verious sources (healthy plants, plants inoculated with *Pseudomonas solanacearum* strains: compatible , incompatible and the hrp- mutant, after different incubetion times). Such binding may be estimeted by retardation gel enelysis using, for example, the 74 bp region and several sub-fragments thus enabling identification of discrete sequences within the BRE region which are useful in providing genetic constructs comprising inducible disease resistance genes.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Hypersensitivity related gene

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2778 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Tobacco

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1413..2417

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TGATTCTTAT GAAATTGATT TTATTAAACC TTCCTACATT TTTAATAATA ATTTAATAGA   480

CAAAATTTTA TTAATTTTAA ATATTAAATA TTAAAAATTA GTAGCATATA AGGTATTATA   540

GTCCAAAAAA TAGCTTATTA CAGTTACGTA CTCTTCCTAT GAGTTCTTTC GTTTAATAAT   600

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CTAATTTTTG ATTGCAGTCC GAAAACTATC TAGTCTATGC TATTTTCACT TTTCTAAACT   900

CCCTGCCACT GTATGCTTTC ATTGGATTAA CCTTTAACCA CACAAATATT TTAAAGAGTA   960

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ACTTTCCTAA TGCTAGTGCT CTTCCCATTA TCTAAACTCC AAAATTTTGT AAAATTCTTT  1260

GAACCTTCCT TTAAACTACC ACAAATTTTC TTATCCTTTC CTATCTCACC ATTATAAATA  1320

GCCACGCACA TGCAAACCAA AGGTACACAC TAAACAAACT TCATTCTTCA AATTACTGAT  1380

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Asp Arg Thr Trp Thr Gly Pro Pro Glu Val Lys Phe Met Ala Glu Pro
     25                  30                  35

GTC CCA CCC CAT GAC TAC TTC ATC GAC GGC GTT GCC GTC AAA GAT GTA     1577
Val Pro Pro His Asp Tyr Phe Ile Asp Gly Val Ala Val Lys Asp Val
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CGA AAC GAC AAT TCC GCC AGC AAG CTT CCC GTC ATT CTT CAC TTC CAA     1673
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GGG TTA GCT TTA CCA GTA GGG AGC AAC AAG GAT CAT CAA ATA ACA TGT      2153
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TTGAGATTCA TGAAATAATA TCTTTAAATT TCGAATGTCA ATAAGTCCAG AAATTGAAAT    2679

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 335 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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        35                  40                  45

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Leu Arg Ile Tyr Leu Pro Glu Arg Asn Asp Asn Ser Ala Ser Lys Leu
65                  70                  75                  80

Pro Val Ile Leu His Phe Gln Gly Gly Gly Phe Cys Val Ser His Ala
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Asp Phe Asn Arg Val Phe Leu Ile Gly Asp Ser Ser Gly Gly Asn Ile
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Val His Gln Val Ala Val Lys Ala Gly Glu Glu Asn Leu Ser Pro Met
            180                 185                 190

Arg Leu Ala Gly Ala Ile Pro Ile His Pro Gly Phe Val Arg Ser Tyr
        195                 200                 205

Arg Ser Lys Ser Glu Leu Glu Gln Glu Gln Thr Pro Phe Leu Thr Leu
    210                 215                 220

Asp Met Val Asp Lys Phe Leu Gly Leu Ala Leu Pro Val Gly Ser Asn
225                 230                 235                 240

Lys Asp His Gln Ile Thr Cys Pro Met Gly Glu Ala Ala Pro Ala Val
                245                 250                 255

Glu Glu Leu Lys Leu Pro Pro Tyr Leu Tyr Cys Val Ala Glu Lys Asp
            260                 265                 270

Leu Ile Lys Asp Thr Glu Met Glu Phe Tyr Glu Ala Met Lys Lys Gly
        275                 280                 285

Glu Lys Asp Val Glu Leu Phe Ile Asn Asn Gly Val Gly His Ser Phe
```

290 295 300

Tyr Leu Asn Lys Ile Ala Val Arg Met Asp Pro Val Thr Gly Ser Glu
305 310 315 320

Thr Glu Lys Leu Tyr Glu Ala Val Ala Glu Phe Ile Asn Lys His
325 330 335

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 93 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTTGCCAAAA TGGTTCATGA AAAGCAAGTG ATAGAGGAAG TATCCGGCTG GCTTAGAGTT 60

TTCGGGGTAG GTCAGTCCCT TATGTTACGT CCT 93

Claims

1. A recombinant DNA sequence including a region comprising the nucleotide sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

2. A recombinant DNA sequence including a region comprising nucleotides 1413 to 2417 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

3. A recombinant DNA sequence including a region comprising nucleotides 1 to 1341 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

4. A recombinant DNA sequence including a region comprising nucleotides 1 to 651 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

5. A recombinant DNA sequence including a region comprising nucleotides 652 to 1341 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

6. A recombinant DNA sequence including a region comprising nucleotides 1195 to 1341 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or recombinant sequence comprising a part of said region or said equivalent.

7. A recombinant DNA sequence including a region comprising nucleotides 1195 to 1268 of the sequence depicted in SEQ ID No. 1 or a functional equivalent thereof, or a recombinant sequence comprising a part of said region or said equivalent.

8. A recombinant DNA sequence comprising at least one region or part or equivalent thereof, according to any one of claims 3 to 7, wherein said region or part or equivalent is located on the 5' side of, and is op-

erably linked to, a protein-encoding sequence of e heterologous gene or to a sequence comprising nucleotides 1413 to 2417 of the sequence depicted in SEQ ID No. 1 or e functional equivalent thereof.

9. A recombinant DNA sequence according to the preceding claim, wherein e translation enhancing sequence is present between the region or part or equivelent thereof, end the protein-encoding region of the DNA sequence 3' thereto.

10. A recombinent DNA sequence according to either of cleims 8 or 9, wherein the heterologous gene is a selectable or screenable marker gene or a gene, the product of which is capable of conferring resistance or tolerance to at least one of the following: insects, herbicides, fungi, bacterie and viruses.

11. A recombinent DNA sequence eccording to eny preceding claim, wherein one or more nucleotides heve been edded to, removed from or substituted in the recombinent sequence without substantially effecting the function or amino acid encoding capability thereof.

12. Recombinant DNA according to eny one of cleims 1 to 11, which is modified in thet codons which are preferred by the organism into which the recombinent DNA is to be inserted are used so thet expression of the thus modified DNA in the said organism yields substantielly similar protein to that obtained by expression of the unmodified recombinent DNA in the orgenism in which the protein-encoding components of the recombinant DNA are endogenous.

13. A DNA sequence which is complementery to one which, under stringent conditions, hybridizes to the recombinant DNA sequence according to any one of claims 1 to 12.

14. A DNA vector comprising a recombinant DNA sequence according to eny one of claims 1 to 12, or e DNA sequence according to claim 13.

15. Protein obteined by expression of the DNA eccording to eny one of cleims 1 to 13.

16. Protein heving the emino ecid sequence depicted in SEQ ID No. 2, or e functional equivelent of seid sequence.

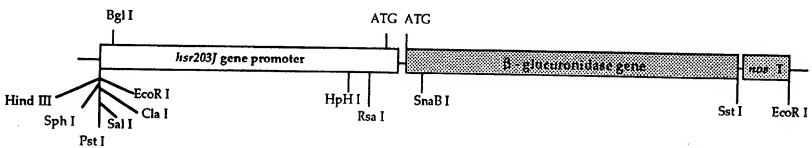
17. A micro-orgenism or plent cell or protoplast which hes been transformed with recombinent DNA eccording to any one of cleims 1 to 11, or e DNA sequence according to claim 12.

18. A plant, the genome of which comprises e vector according to claim 14, in which plant the recombinent DNA is expressed.

19. A plent eccording to the preceding cleim selected from the group consisting of: tometoes, peppers, mangoes, peaches, apples, pears, strawberries, bananas, melons, canole, sunflower, tobacco, sugar beet, wheet, berley, rice, corn, cotton, potato, cerrot, lettuce, cabbege end onion.

20. The progeny or seeds of plants eccording to either of cleims 18 or 19, and the seeds end progeny of said progeny.

A. pHG 21



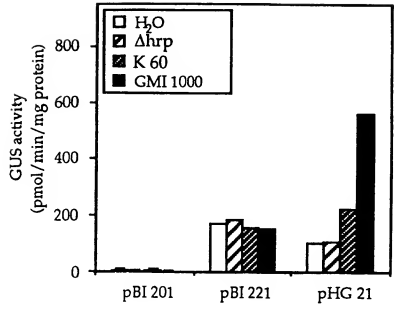
B.

Translational fusion sequence :...TTT GCC AAA ATG GTT CAT GAA AAG CAA GTG ATA GAG
GAA GTA TCC GGC TGG CTT AGA GTT TTC GGG GTA GGT CAG TCC CTT ATG TTA CGT CCT...

20

Fig. 1

Fig. 2


H2O
Δhrp
K 60
GMI 1000
GUS activity
(pmol/min/mg protein)
800
600
400
200
0
pBI 201
pBI 221
pHG 21

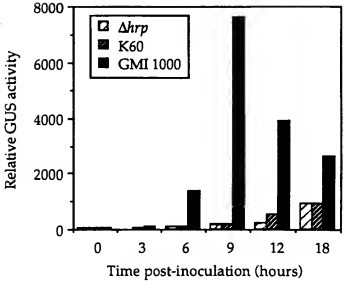

8000
6000
4000
2000
0
Relative GUS activity
Δhrp
K60
GMI 1000
0
3
6
9
12
18
Time post-inoculation (hours)

Fig. 3

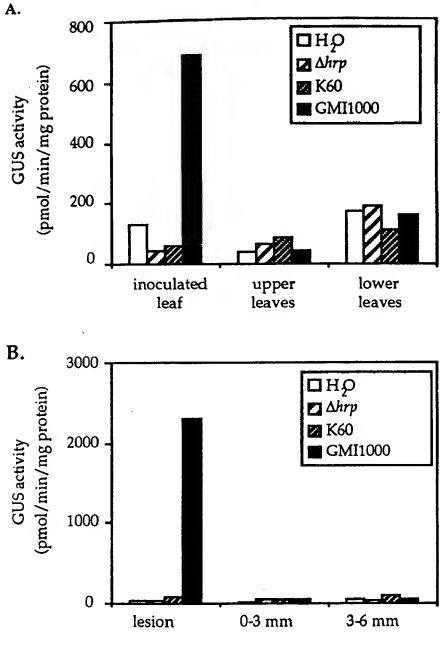

A.
800
600
400
200
0
GUS activity
(pmol/min/mg protein)
H2O
Δhrp
K60
GMI1000
inoculated leaf
upper leaves
lower leaves
B.
3000
2000
1000
0
GUS activity
(pmol/min/mg protein)
H2O
Δhrp
K60
GMI1000
lesion
0-3 mm
3-6 mm
Tissue samples

Fig. 4

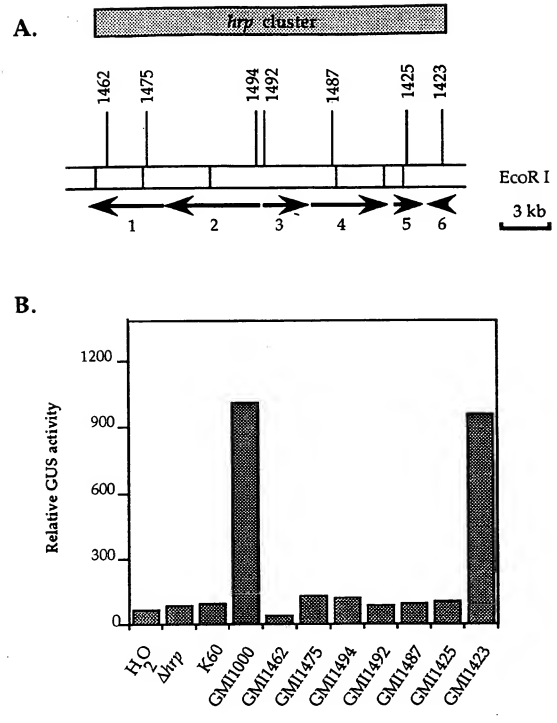

A.
hrp cluster
1462
1475
1494
1492
1487
1425
1423
EcoR I
3 kb
1
2
3
4
5
6
B.
Relative GUS activity
1200
900
600
300
0
H2O2
Δhrp
K60
GMI1000
GMI1462
GMI1475
GMI1494
GMI1492
GMI1487
GMI1425
GMI1423

Fig. 5

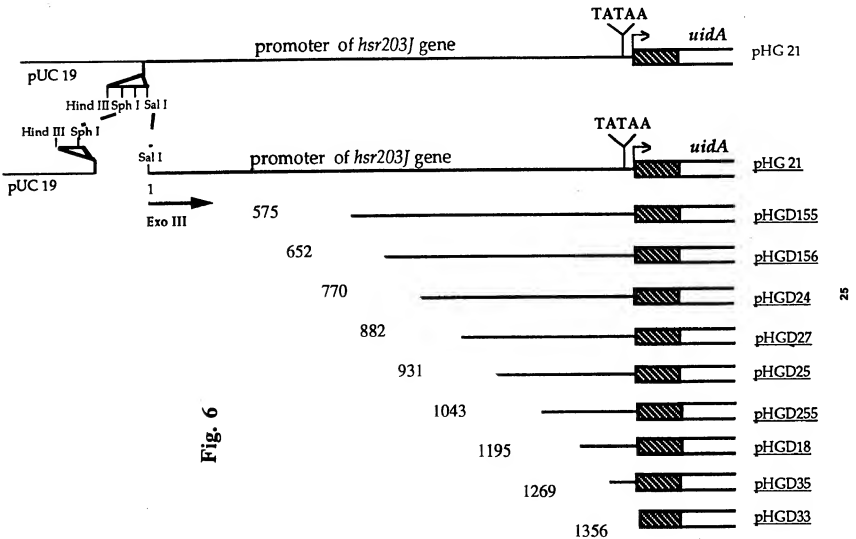

TATAA
uidA
promoter of hsr203J gene
pHG 21
pUC 19
Hind III Sph I Sal I
Hind III Sph I
Sal I
pUC 19
1
Exo III
575
652
770
882
931
1043
1195
1269
1356
pHGD155
pHGD156
pHGD24
pHGD27
pHGD25
pHGD255
pHGD18
pHGD35
pHGD33

Fig. 6

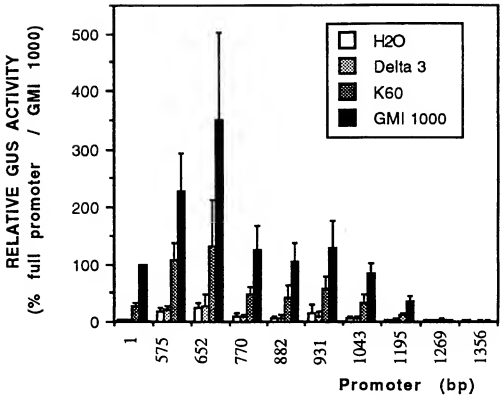

RELATIVE GUS ACTIVITY
(% full promoter / GMI 1000)
500
400
300
200
100
0
H2O
Delta 3
K60
GMI 1000
1
575
652
770
882
931
1043
1195
1269
1356
Promoter (bp)

Fig. 7